Leading Edge
Review
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Invasion of Red Blood Cells by Malaria Parasites

Alan F. Cowman[1,*] and Brendan S. Crabb[1,*]
[1]The Walter and Eliza Hall Institute of Medical Research, Melbourne 3050, Australia
*Contact: cowman@wehi.edu.au (A.F.C.); crabb@wehi.edu.au (B.S.C.)
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The malaria parasite is the most important member of the Apicomplexa, a large and highly successful phylum of intracellular parasites. Invasion of host cells allows apicomplexan parasites access to a rich source of nutrients in a niche that is largely protected from host defenses. All Apicomplexa adopt a common mode of host-cell entry, but individual species incorporate unique features and utilize a specific set of ligand-receptor interactions. These adhesins ultimately connect to a parasite actin-based motor, which provides the power for invasion. While some Apicomplexa can invade many different host cells, the disease-associated blood-stage form of the malaria parasite is restricted to erythrocytes.

Introduction

It has been over 100 years since the discovery that malaria, a scourge of humanity since antiquity, is caused by infection with the protozoan parasite *Plasmodium*. There are many *Plasmodium* species that infect animals ranging from humans and other mammals to birds and lizards; they represent an extremely successful group of parasites. In humans, the most severe form of malaria is caused by *Plasmodium falciparum*, and at least one-third of the world's population is at risk of infection, with over 300 million people developing clinical disease each year and at least 2 million deaths (Snow et al., 2005). Other *Plasmodium* spp. including *P. vivax* infect humans and cause considerable morbidity in those populations where it is endemic. The overall toll caused by malaria has changed little since the 1950s, when the first mass initiatives based on elimination of the mosquito vector and antimalarial prophylaxis were enacted. While there have been major reductions in mortality and morbidity in some areas, such as South Asia, malaria remains a major pediatric killer in many parts of sub-Saharan Africa, which bears the greatest burden of disease. It is increasingly clear that a greater understanding of the host-parasite interaction would assist in the development of new treatments and, most importantly, a vaccine for long-term sustainable reduction in the global burden of malaria.

In contrast to many other pathogenic organisms, *Plasmodium* spp. can efficiently infect their hosts by rapidly gaining entry to cells using their own invasion apparatus to identify, penetrate, and establish themselves. Host-cell entry by *Plasmodium* represents an obligatory and parasite-specific process, an Achilles' heel that can be exploited for development of new treatments. In this review, we address the mechanisms that these parasites utilize for erythrocyte invasion with strong emphasis on *P. falciparum*, but we draw on lessons learned from related parasites.

Plasmodium and Apicomplexan Parasites

The phylum Apicomplexa includes parasites of human and veterinary importance such as *Toxoplasma*, *Theileria*, *Eimeria*, *Babesia*, and *Cryptosporidium*. These parasites have complex life cycles, with some, including *Toxoplasma*, *Eimeria*, and *Cryptosporidium*, passing directly between vertebrate hosts. In contrast, the life cycle of others, including *Plasmodium* (Figure 1), involves an arthropod vector that transmits the parasite to a vertebrate host during blood feeding. Regardless of their host or mode of transmission, all apicomplexan parasites share features including presence of a specialized apical complex (for which the group is named), which is central to the invasion process.

Plasmodium in mammalian hosts is transmitted by female *Anopheles* mosquitoes, and sporozoite forms are injected into the vertebrate host during a blood meal (Figure 1). These extracellular forms rapidly migrate to the liver via the bloodstream and pass through a number of host cells before actively invading hepatocytes. Each invading sporozoite differentiates and divides mitotically into thousands of liver merozoites that, when released, invade erythrocytes, thus beginning the asexual blood-stage lifecycle of this parasite. Although their morphology differs somewhat, merozoites share characteristics with other invasive forms of *Plasmodium*, the sporozoite and insect ookinete form, as well as the invasive forms of other apicomplexan parasites such as the *T. gondii* tachyzoite. These common features, which include a polarized morphology and apical organelles that secrete their contents during host-cell invasion (Figure 2), underlie a common strategy for gaining entry into their respective host cells. Invasion into the erythrocyte by the extracellular merozoite is an essential step, and development of rapid and efficient entry into the host cell has been important in the evolutionary success of these pathogens.

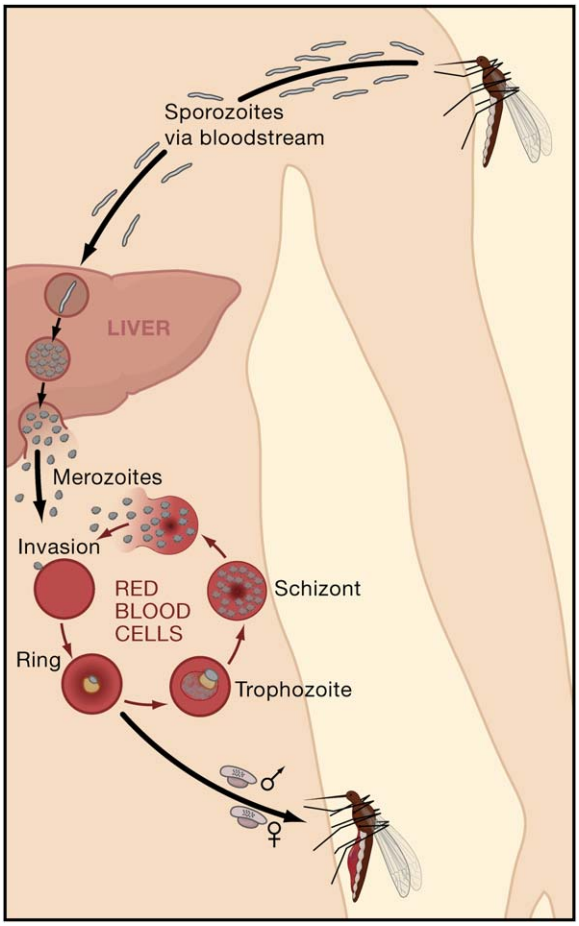


Figure 1. The Life Cycle of *P. falciparum* in the Human Host and the *Anopheles* Mosquito Vector

The *Plasmodium*-infected mosquito injects sporozoite forms into the human host, and these migrate to the liver, where they can pass through Kuppfer cells and invade hepatocytes within which they develop into liver merozoites. These merozoites are released into the bloodstream, where they invade erythrocytes. They develop through ring, trophozoite, and schizont stages, replicating to produce from 16 to 32 daughter merozoites that are released during egress. The free merozoite is then able to invade other erythrocytes to continue the asexual blood-stage life cycle. All clinical symptoms arise during this asexual blood cycle. In the blood, some intraerythrocytic stages develop into male or female gametocytes, the sexual forms of the parasite. These are taken up by the mosquito during feeding, develop into gametes in the insect gut, and fuse to form zygotes. The zygote develops to form an invasive ookinete, which traverses the midgut and transforms into an oocyst from which sporozoites are released that migrate to the salivary glands for injection into a human host during the next blood meal.

General Overview of *Plasmodium* Invasion

To invade the host cell, the parasite must firstly initiate egress from its infected cell, and this process involves disruption of its vacuolar home, known as the parasitophorous vacuole, and the host-cell membrane. Egress of *P. falciparum* involves a sudden increase in intracellular pressure late in the blood-stage cycle, together with biochemical changes that destabilize the infected cell cytoskeleton, and these combine to promote an explosive event effectively dispersing the nonmotile merozoites (Glushakova et al., 2005) (Figure 3). The underlying cause of the increased pressure and destruction of the cytoskeleton preceding rupture is not completely clear. With respect to the latter, however, it would appear that proteases are involved (Salmon et al., 2001) and that this occurs in a two-step process such that destruction and rupture of the internal vacuolar membrane occur distinctly and just prior to that of the erythrocyte membrane (Wickham et al., 2003). Candidate proteases include members of the serine repeat antigen (SERA) family that localize to the parasitophorous vacuole (Hodder et al., 2003; Miller et al., 2002b). The SERA family is required in mosquito stages for sporozoite egress from oocysts (a stage of the parasite in the mosquito vector), validating a role in host-cell rupture for proteases in general and this family in particular (Aly and Matuschewski, 2005). After exiting the host, merozoites recognize, attach, and enter erythrocytes, and this process occurs rapidly, probably on the order of 60 s. It is not surprising that this time frame is short and highly efficient, as the antigens on the surface of the extracellular form of the parasite are particularly vulnerable to immune attack.

The initial contact between the merozoite and erythrocyte is a crucial step, as the parasite must distinguish between erythrocytes competent for invasion and other cell types (Figure 4). At a gross level, this recognition and primary adherence is relatively long distance, apparently of low affinity, and reversible (Bannister and Dluzewski, 1990). Primary attachment of the polar merozoite appears to occur at any point on the surface of this parasite stage. Reorientation then ensues (Figure 4), an event that seems to involve the erythrocyte surface wrapping around the merozoite. The purpose of this event is to juxtapose the apical end of the merozoite with the erythrocyte membrane, allowing a much closer interaction.

To enter the cell, a so-called tight junction is formed between the parasite and host membrane. The tight junction moves from the apical to the posterior end of the merozoite in a complex series of events powered by the parasite actin-myosin motor (Keeley and Soldati, 2004). One of the most conspicuous events is the removal or "shedding" of the fuzzy coating covering the merozoite surface, a proteolytic event involving SUB2, a serine protease localized in the apical microneme organelles (Figure 2 and Figure 4) (Harris et al., 2005). As the tight junction moves toward the posterior end the ligands mediating the invasion process must be removed. By analogy with *T. gondii*, it appears likely that, in *P. falciparum*, this may involve the rhomboid family of serine proteases which, unusually, clip proteins from within the phospholipid bilayer (Brossier et al., 2005; Dowse et al., 2005). As the parasite pushes its way into its host cell, it creates a parasitophorous vacuole to seal itself from the host-cell cytoplasm and form an environment hospitable for its development.

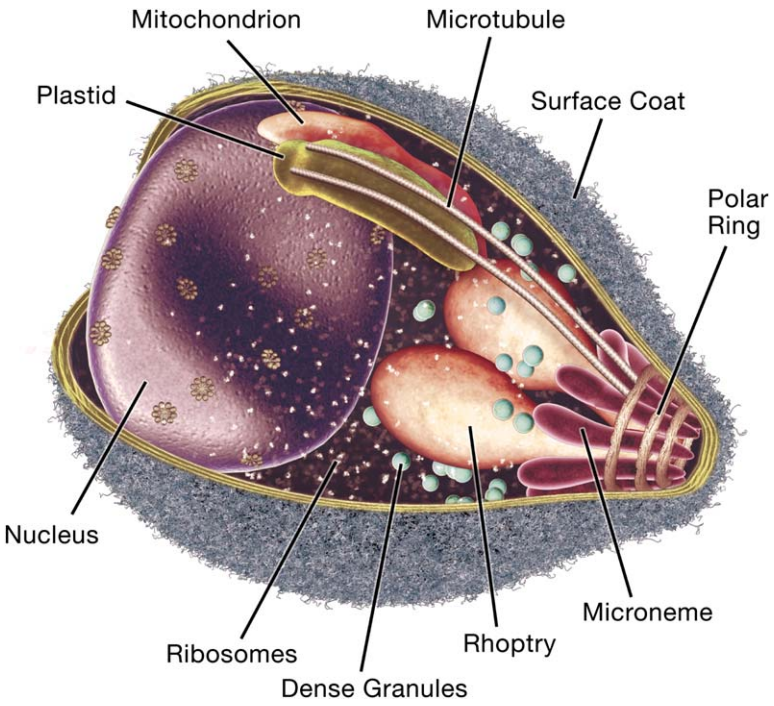


Figure 2. Diagram of a Merozoite, Highlighting Major Organelles and Cellular Structures

Note that the dense granules are seemingly heterogeneous in their contents, and, hence, different dense granules perform different functions. It is possible that the microneme organelles are similarly heterogeneous.

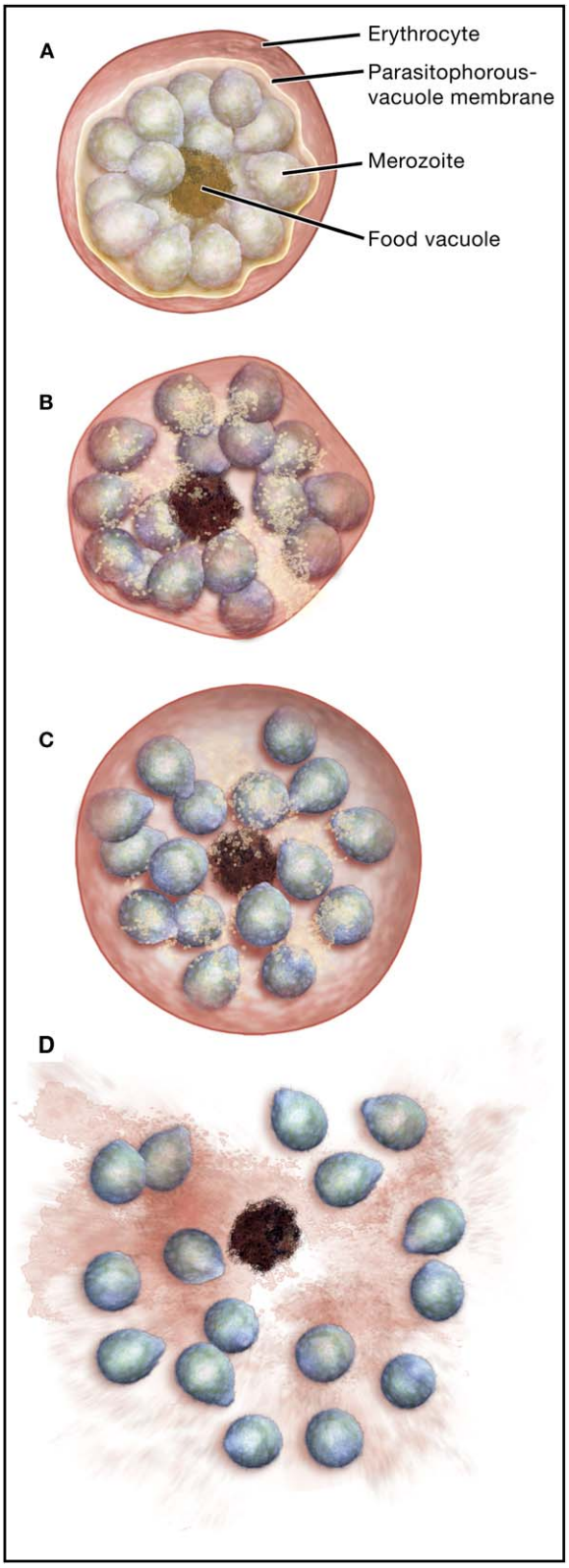
Molecular Basis of Invasion

Primary Contact. The surface coat of merozoites is largely comprised of glycosylphosphatidylinositol (GPI) anchored membrane proteins and their associated partners (Sanders et al., 2005). Currently there are nine GPI anchored proteins known or predicted to be located on the merozoite surface of *P. falciparum*, and all are potential erythrocyte ligands (Table 1). Such GPI-anchored proteins are not necessarily evenly spread over the surface, and some (e.g., Pf38) have a more apical localization consistent with different roles in invasion (Sanders et al., 2005). Besides a GPI anchor, many surface proteins share similarities including cysteine-rich domains that are of potential significance in adherence. These domains include epidermal-growth-factor-like modules at the C terminus of four surface proteins, and "6-cys" modules in Pf12 and Pf38. Other 6-cys family members are also found on the surface of *P. falciparum* gametes and sporozoites (Ishino et al., 2005; van Dijk et al., 2001).

Modeling data has revealed that the structure of the 6-cys proteins resembles that of the surface antigen (SAG) related sequence (SRS) superfamily found on the surface of *T. gondii* tachyzoites previously thought to be restricted to tissue-associated coccidian members of the Apicomplexa phylum (Gerloff et al., 2005). The dual cys-rich domains in the prototype *T. gondii* SRS family member SAG1 dimerize to form a receptor binding site at the tip of the molecule (He et al., 2002). Members of the *Plasmodium* 6-cys family are already recognized as playing adhesive roles on the surface of gametes (van Dijk et al., 2001) and sporozoites (Ishino et al., 2005), and the discovery of three different family members on the surface of blood stages suggests that these also function in this manner. The fact that these proteins possess an SRS-type fold adds weight to this, and perhaps they represent the elusive adhesins that initiate contact with erythrocytes.

MSP-1 is a dominant antigen and the most abundant protein on the surface, is thought to be essential for parasite survival, and is a major vaccine candidate (O'Donnell et al., 2000). MSP-1 has long been considered the best candidate to mediate initial contact to the host erythrocyte, and, while there is some evidence (Goel et al., 2003), at this stage, definitive proof is lacking. Additionally, the important EGF-containing domain, MSP-1_{19}, can be radically altered without affecting invasion, a feature that appears inconsistent with specific binding to an erythrocyte receptor (Drew et al., 2004; O'Donnell et al., 2000). It remains possible that MSP-1, particularly its more N-terminal regions, is involved in early recognition and attachment events, but this remains to be defined.

Nevertheless, MSP-1_{19} in particular is considered an important domain, and its structure has been solved. Key structural features include its overall shape—a compact, flat, almost disc-like structure—and the arrangement of the two EGF domains, which are folded back on one another in a side-by-side arrangement rather than end on end as in other multiple EGF domain proteins (Chitarra et al., 1999; Morgan, 1999; Pizarro et al., 2003). The compact nature of this small (~90 amino acid) region, dependent on 6-disulphide bonds and other close interdomain contacts, places constraint on the molecule such that it appears difficult to generate individual point mutations in the face of substantial immune pressure (Drew et al., 2004; O'Donnell et al., 2001). A definitive function



for this leading vaccine candidate molecule remains elusive.

Peripheral proteins are also candidates for binding to erythrocyte receptors. These proteins are secreted into the parasitophorous vacuole of schizont-stage parasites and bind to the surface of developing merozoites, at least to some degree, via an interaction with a GPI-anchored protein such as MSP-1 (Pachebat et al., 2001). Most peripheral proteins belong to one of three broad families of proteins, the MSP-3/-6 group, the MSP-7 family, and the SERA protease family (Table 1). Other peripheral proteins include the acid basic repeat antigen (ABRA) and Pf41, another 6-cys family member. Despite there being many members, it appears that only a few peripheral proteins, including MSP-7 family members and Pf41, are strongly associated with the surface and hence are likely to represent core structural components (Sanders et al., 2005). These are the best candidates to mediate primary contact, but a role for more loosely associated proteins such as MSP-3 and MSP-6 or indeed other unidentified proteins cannot be ruled out. It remains to be established which of the core surface proteins, whether membrane associated or peripheral, have adhesive roles in the initial recognition and interaction of erythrocytes by merozoites. The difficulties in dissecting this issue relate to the likelihood that adhesion is of low affinity and probably requires a high concentration of correctly oligomerized surface ligands in a manner that is difficult to reproduce in vitro.

In contrast to the merozoite surface proteins, there is clear evidence that a number of proteins stored in the apical organelles bind to specific receptors on the erythrocyte and are directly involved in invasion. While some of these proteins have orthologs throughout *Plasmodium* spp., they are generally not conserved more broadly within Apicomplexa. However, there are some proteins, such as AMA-1, that are highly conserved throughout the phylum. AMA-1 is essential for invasion of *P. falciparum* and *T. gondii* (Triglia et al., 2000; Mital et al., 2005). PfAMA1 is translocated to the merozoite surface before invasion commences, and antibodies specifically block this entry process, although they do not interfere with initial attachment (Mitchell et al., 2004). PfAMA1 is also expressed in the invasive sporozoite form of the parasite, and PfAMA-1 is essential for sporozoite invasion of hepatocytes (Silvie et al., 2004). In *T. gondii*, TgAMA1 plays no role in initial attachment to the host cell but precedes secretion of the rhoptries, suggesting a role in establishment of the apical interaction and regulation of rhoptry secretion (Mital

Figure 3. Egress of *Plasmodium* Merozoites

(A) Mature schizont-stage parasites contain 16 or so fully formed merozoites.

(B) The parasitophorous-vacuole membrane ruptures first, apparently due to the action of proteases, and the merozoites are free within the intact erythrocyte.

(C and D) Swelling of the erythrocyte and degradation of the cytoskeleton (C) leads to an explosive lysis and scattering of the free merozoites and vesiculation of the host-cell membrane (D).

et al., 2005). This is consistent with evidence showing that inhibition of PfAMA1 function in *P. falciparum* using a specific antibody did not prevent initial attachment but blocked reorientation of the merozoite (Mitchell et al., 2004). There is still much to be determined with respect to the role of AMA-1 in merozoite invasion, but it appears to be required to establish the apical interaction through parasite adhesins located initially at the neck of the rhoptries and in the micronemes. It likely represents a key link between the weak initial contact involving MSPs and irreversible tight associations formed with microneme proteins. Indeed, it has recently been shown that AMA-1 interacts with at least one other protein (RON4) in *T. gondii* tachyzoites at the moving junction during invasion, and homologs have been identified in *P. falciparum*, suggesting a conserved complex (Alexander et al., 2005; Lebrun et al., 2005).

The ectodomain of PfAMA-1, a classical type 1 membrane protein, is largely comprised of three domains: a relatively N-terminal domain I, a central domain II, and a C-terminal domain III (Bai et al., 2005; Nair et al., 2002; Pizarro et al., 2005). Structural studies reveal that both domains I and II possess a PAN (or apple domain) fold (Pizarro et al., 2005). This was not recognized from the primary sequence, and it is significant because these folds in other proteins have been implicated in receptor binding. It is highly likely that structural information will ultimately lead to great insight into AMA-1 function. For example, polymorphic residues that have accumulated on AMA-1 in response to selection pressure appear to be concentrated around a deep, highly conserved groove (Bai et al., 2005). There is obviously a strong likelihood that this region fulfils an important role in invasion.

Secondary Interactions. After the parasite reorientates, it must activate the invasion process, and this probably involves direct interaction of ligands at the apical end with erythrocyte receptors; these adhesins have not been defined, but two protein families, the Duffy binding-like (DBL) protein family (Miller et al., 2002a) and *P. falciparum* reticulocyte binding protein homolog (PfRh or PfRBL), are prime candidates (Rayner et al., 2001; Duraisingh et al., 2003b; Stubbs et al., 2005; Triglia et al., 2005).

The DBL proteins include EBA-175 (Camus and Hadley, 1985), EBA-140 (also known as BAEBL) (Maier et al., 2003; Mayer et al., 2001; Thompson et al., 2001), and EBA-181 (also known as JSEBL) (Gilberger et al., 2003b). Another DBL gene family member, *eba-165* (also known as PEBL) of *P. falciparum*, appears not to be expressed as a functional protein. These proteins are orthologs of DBL proteins identified in *P. vivax*. The cysteine-rich dual DBL domains found toward the N terminus of EBA-175 mediates binding to its cognate receptor, and it is likely that similar domains in EBA-140 and EBA-181 also play receptor binding roles. The cytoplasmic tail of the DBL proteins does not appear to be directly linked to the actin-myosin motor (Gilberger et al., 2003a); this function is probably performed by a thrombospondin anonymous repeat protein (TRAP) homolog (see below) (Baum et al., 2005).

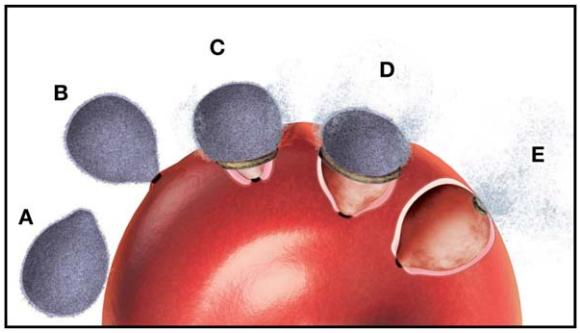


Figure 4. Merozoite Invasion of Erythrocytes
Invasion involves an initial "long-distance" recognition of surface receptors (A) followed by a reorientation process whereby these low-affinity contacts are maintained. Once the apical end is adjacent to the erythrocyte (B), a tight junction is formed involving high-affinity ligand receptor interactions. This tight junction then moves from the apical to posterior pole (C and D) powered by the parasite's actin-myosin motor. The surface coat is shed at the moving junction by a serine protease, or "sheddase." Upon reaching the posterior pole, the adhesive proteins at the junction are also proteolytically removed (E), this time by a resident protease, most likely a rhomboid, in a process that facilitates resealing of the membrane. By this process, the parasite does not actually penetrate the membrane but invades in a manner that creates a parasitophorous vacuole.

The PfRh proteins were identified as homologs of rhoptry proteins in *P. yoelii* (Preiser et al., 2002) and *P. vivax* (Galinski et al., 1992) and have been implicated in determining the specificity of host-cell invasion. *P. vivax* is a parasite of humans that preferentially invades reticulocytes, and it expresses two homologs of the Py235 family, PvRBP1 and PvRBP2 (Galinski et al., 1992). These proteins bind to reticulocytes but not normocytes, suggesting that they are responsible for the host-cell preference of this species. It was therefore not surprising that *P. falciparum* also expresses an equivalent protein family, which consists of four members, PfRh1 (PfRBP1), PfRh2a (PfRBP2a), PfRh2b (PfRBP2b), and PfRh4 (PfRBP4); a fifth, PfRh3 (PfRBP3), does not appear to be expressed as a protein (Duraisingh et al., 2003b; Rayner et al., 2001; Stubbs et al., 2005; Triglia et al., 2001, 2005).

DBL proteins bind erythrocytes in a sialic-acid-dependent manner as neuraminidase treatment of the host cell ablates binding. EBA-175 and EBA-140 bind to glycophorin A (Orlandi et al., 1992) and C, respectively (Maier et al., 2003), and while sialic acid on these receptors is essential for binding, the protein backbone is also important for specificity. EBA-181 and PfRh1 also bind to glycosylated erythrocyte receptors, although their identity is currently unknown (Gilberger et al., 2003b; Rayner et al., 2001). In contrast, there is no evidence that PfRh2a directly binds to erythrocytes (Duraisingh et al., 2003b). PfRh2b and PfRh4 have also not been shown to directly bind erythrocytes; however, they are involved in merozoite invasion as disruption of the corresponding gene causes these parasites to change the receptor they use for invasion (Duraisingh et al., 2003b; Stubbs et al., 2005).

Table 1. Properties of Some Important Merozoite Proteins in *P. falciparum*

Name	Accession Number	KO	Features/Structure
GPI-Anchored (Known or Putative) Surface Proteins			
MSP-1	PFI1475w	N	Putative band 3 ligand; processing and removal of bulky complex essential for invasion; C-terminal double EGF domains functionally redundant across divergent molecules/compact side-by-side arrangement of EGFs
MSP-2	PFB0300c	N[a]	Highly polymorphic; two major alleles functionally identical; potential species-specific function
MSP-4	PFB0310c	N[a]	C-terminal single EGF domain
MSP-5	PFB0305c	Y[a]	Not required for invasion; homolog of MSP-4
MSP-10	MAL6P1.221	N[a]	Surface and apical appearance; C-terminal double EGF module
Pf12	PFF0615c	ND	Member of 6-cys family; surface-only/6-cys domains modeled as similar to *T. gondii* surface protein SAG1
Pf38	PFE0395c	ND	Member of 6-cys family; surface and apical appearance
Pf92	PF13_0338	N[a]	Cysteine-rich surface protein
Pf113	PF14_0201	N[a]	Putative surface protein
Microneme Proteins			
AMA-1	PF11_0344	N	Partial complementation between *P. falciparum* and rodent species; antibodies and peptides block invasion/merozoite reorientation/ PAN domains, polymorphisms surround conserved hydrophobic pocket
EBA-140/BAEBL	MAL13P1.60	Y	Binds glycophorin C (Gerbich antigen)
EBA-175	PF07_0128	Y	Binds to glycophorin A; disruption in *P. falciparum* (W2mef) leads pathway switch to Rh4-dependent invasion/"handshake" association between region II dimers creates grooves for GlyA glycan binding
EBA-181/JESEBL	PFA0125c	Y	Binds trypsin-resistant receptor W on RBCs
EBL1	PFD1145c	ND	No known function
MTRAP	PF10_0181	N	Motor-associated protein
ASP	PFD0295c	ND	Contains "sushi" domain, hence the name apical sushi protein, ASP; putative micronemal GPI-anchored protein
SUB2	PF11_0381	N[a]	Subtilisin-like serine protease; MSP-1/AMA-1-processing "sheddase"
Peripheral Surface Proteins			
ABRA	PFL1385c	Y[b]	No known function but putative protease; probably nonessential as not trafficked correctly in MSP-3 knockout
S-antigen	PF10_0343	N[a]	Chr10 locus; no known function
GLURP	PF10_0344	ND	Chr10 locus; no known function
MSP3	PF10_0345	Y	Chr10 locus; abundant but weakly associated with surface/elongated tetrameric molecule; helical heptad repeats predicted to form coiled coil
MSP6	PF10_0346	Y[a]	Chr10 locus "MSP3-like" protein; weakly associated with MSP1
H101	PF10_0347	Y	Chr10 locus "MSP3-like" protein; no known function
H103	PF10_0351	Y	Chr10 locus "MSP3-like" protein; no known function
MSP7	PF13_0197	Y[a]	MSP-1 binding protein; strongly associated; disruption in *P. berghei* suggests role in normocyte (not reticulocyte) invasion
MSP7-like	MAL13P1.174	ND	Putative MSP1 binding protein
MSP7-like	PF13_0196	ND	Putative MSP1 binding protein, associated with detergent-resistant membranes

Table 1. Continued

Name	Accession Number	KO	Features/Structure
Pf41	PFD0240c	ND	Member of 6-cys family; apical end of merozoites
SERA3	PFB0350c	Y	Cysteine protease domain with active-site serine
SERA4	PFB0345c	Y[a]	Cysteine protease domain with active-site serine; schizont expression
SERA5	PFB0340c	N	Cysteine protease domain with active-site serine; strong schizont expression
SERA6	PFB0335c	N	Cysteine protease domain with active-site cysteine; schizont expression
Rhoptry Proteins			
RAMA	MAL7P1.208	N[a]	GPI anchored, possibly associating with rhoptry complexes
RAP1	PF14_0102	Y	Low-molecular-weight rhoptry complex, nonessential, truncation disrupts trafficking of RAP2 and 3
RAP2	PFE0080c	Y[b]	Low-molecular-weight rhoptry complex; nonessential
RAP3	PFE0075c	Y[b]	Low-molecular-weight rhoptry complex; nonessential
RhopH1(2)	PFB0935w	ND	High-molecular-weight rhoptry complex; CLAG2
RhopH1(3.1)	PFC0110w	ND	High-molecular-weight rhoptry complex; CLAG3.1
RhopH1(3.2)	PFC0120w	ND	High-molecular-weight rhoptry complex; CLAG3.2
RhopH1(9)	PFI1730w	Y	High-molecular-weight rhoptry complex; CLAG9
RhopH2	PFI1445w	ND	High-molecular-weight rhoptry complex
RhopH3	PFI0265c	N[a]	High-molecular-weight rhoptry complex
Rhoptry Neck Proteins			
Rh1	PFD0110w	Y	Binds red blood cells via receptor Y
Rh2a	PF13_0198	Y	No demonstrated function
Rh2b	MAL13P1.176	Y	Involved in invasion pathway through receptor Z
Rh3	PFL2520w	Y	Probable transcribed pseudogene
Rh4	PFD1150c	Y	Differential expression allows invasion-pathway switching
Rh5	PFD1145c	N	No known function

Proteins are grouped according to their localization.
[a] Unpublished data. N = KO attempt unsuccessful; Y = KO generated; ND = not done.
[b] These do not represent genetic deletions but are functional knockouts due to the incorrect localization of the protein in another knockout background.

The dual DBL domains of *P. falciparum* EBA-175 (known as F1 and F2 or collectively as region II) consist of an all-helical domain capable of forming dimers in a ''handshake'' arrangement, and, by doing so, interdomain channels are established that have receptor binding potential (Tolia et al., 2005). Region II binds glycophorin A, making contacts with both protein backbone and terminal sialic-acid side chains. Cocrystallization of this domain with a sialated glycan, together with mutational studies, has revealed that this channel is indeed the likely receptor binding cleft (Tolia et al., 2005).

Many other proteins in *Plasmodium* species possess DBL domains, including the virulence-related protein PfEMP-1 as well as other DBL proteins. *P. vivax* DBL is a key protein in this regard, as is its ortholog in the related primate parasite *P. knowlesi*. These proteins both bind the Duffy antigen on human erythrocytes and are the prototype molecules by which the *Plasmodium* DBL family is named. The structure of the single DBL domain in the *P. knowlesi* protein has recently been solved (Singh et al., 2006). This protein consists of a predominantly α-helical fold similar to that of the EBA-175 DBLs, but the nature of receptor binding appears to be completely different in this domain. *P. knowlesi* DBL does not dimerize—the EBA-175 dimer interface residues are absent here (and, incidentally, they are also absent in PfEMP-1 DBLs)—and so a receptor binding groove is not formed in this protein. In contrast, receptor binding in the *P. knowlesi* DBL appears to occur on one of its exposed faces. Interestingly, residues that line this face are largely invariant, while amino acids on the opposite face are more divergent. It has been proposed that the binding site is protected

from immune-driven mutation because the period of time between release of the DBP from the micronemes and the formation of a tight association with the Duffy antigen receptor is very short (the "just-in-time" hypothesis of ligand exposure) and is not sufficient to allow the interaction of antibodies with this region (Singh et al., 2006).

While the DBL and PfRh proteins are important in merozoite invasion, it is clear that they are not essential, as the gene for all can be disrupted in different *P. falciparum* lines without an obvious effect on blood-stage growth rates (Duraisingh et al., 2003a, 2003b; Gilberger et al., 2003b; Maier et al., 2003; Stubbs et al., 2005; Triglia et al., 2005). However, they each mediate specific routes of invasion through independent receptors, termed invasion pathways, providing the merozoite with a broad array of ligands. This redundancy in invasion appears to underlie the different patterns of receptor usage or invasion pathways demonstrated in strains of *P. falciparum* both in the laboratory and directly in field isolates (Duraisingh et al., 2003b). Differential expression of the PfRh proteins occur in *P. falciparum*, and this could explain in part the different pattern of invasion pathways used in parasite strains to provide a mechanism of phenotypic variation for invasion into erythrocytes (Duraisingh et al., 2003b). In *P. yoelii*, differential transcription of *Py235* genes has been demonstrated in different merozoites within a single schizont, suggesting a mechanism to vary the particular proteins used for erythrocyte invasion in individual invading merozoites (Preiser et al., 2002). Also, some strains of *P. falciparum* are able to switch their pattern of receptor utilization for erythrocyte invasion, and this involves activation of PfRh4 expression (Stubbs et al., 2005).

Multiple parasite ligands and variation in expression of the PfRh family in *P. falciparum* have important implications for the parasite (Duraisingh et al., 2003b). Merozoite invasion requires sufficient ligand-receptor interactions on the erythrocyte surface, and there are circumstances where receptor availability is limited. First, the molecules on the erythrocyte are highly polymorphic in the human population (Miller et al., 2002a). Second, different erythrocyte receptors such as glycophorin A are not present or vary in their quantity on the surface of this cell during the cell's lifespan. Third, host immune responses to the parasite ligands could block an invasion pathway. Multiple parasite ligands and phenotypic variation of PfRh protein expression provide a mechanism for the parasite to maximize fitness by the presence of merozoites within the population that express different dominant invasion pathways (Duraisingh et al., 2003b; Stubbs et al., 2005).

Calcium and Other Mediators of Invasion. Once apical interaction has occurred, the invading merozoite establishes a tight junction involving release of additional proteins from the micronemes and rhoptry organelles. In *T. gondii*, microneme secretion is regulated by a calcium-release pathway, and it may be triggered by apical interaction during invasion of the host cell (Lovett and Sibley, 2003). While this may be true for other Apicomplexa such as *P. falciparum*, it is clear that components of micronemes, such as PfAMA1, have been translocated to the surface of this invasive cell before or during egress from the erythrocyte (Healer et al., 2002; Mitchell et al., 2004). It is possible that there are distinct subgroups of micronemes that are released at different times before and during merozoite invasion in *P. falciparum* (Figure 2). However, the invasion process is clearly Ca^{2+} dependent as has been shown for *T. gondii* (Lovett and Sibley, 2003). A calmodulin-like domain (calcium-dependent) protein kinase 1 (CDPK1) has been identified in *T. gondii* that can be inhibited by KT5926, and this prevents attachment, suggesting that it is required for signaling secretion of micronemes and formation of the tight junction by release of Ca^{2+} (Dobrowolski et al., 1997; Kieschnick et al., 2001). A CDPK1 homolog is present in *P. falciparum*, suggesting that it may be a key element in signaling activation of invasion (Moskes et al., 2004).

Once apical attachment is established in *P. falciparum*, the parasite may discharge mediators into the erythrocyte to assist in invasion; however, none has yet been identified. In *T. gondii*, small secretory vesicles termed evacuoles are secreted into the host-cell cytoplasm, and these contain parasite proteins for establishment of the parasitophorous vacuole (Hakansson et al., 2001). While transfer of specific evacuoles into the erythrocyte has not been described during *P. falciparum* invasion, electron micrographs of invading merozoites suggest transfer of some material (Aikawa and Miller, 1983). However, it does appear that host components can be used, as entry of *P. falciparum* into erythrocytes requires signaling through erythrocyte β2-adrenergic receptors via heterotrimeric GTP binding proteins (G proteins) (Harrison et al., 2003).

Connections to the Motor. In *Plasmodium* merozoites, the protein or proteins that link to the actin-myosin motor have not been specifically identified, but, in the sporozoite forms that invade liver cells, TRAP appears to provide the crucial link from the extracellular adhesins to the parasite cytoskeleton (Kappe et al., 1999; Sultan et al., 1997). There are a number of TRAP like proteins in apicomplexan parasites containing both integrin A-like domains (A domains) and/or thrombospondin type I repeats (TSRs). The *Plasmodium* TRAP and circumsporozoite protein (CSP) are adhesins mediating binding to receptors such as glycosaminoglycans, and they are both involved in internalization of the sporozoite into liver cells. In *T. gondii*, TgMIC2 possesses both TSR and A domains, and it is important in invasion and gliding motility and has been shown to directly link to the actin-myosin motor through binding of its short cytoplasmic tail to aldolase that then interacts with F actin (see Figure 5) (Jewett and Sibley, 2003). Current evidence suggests that the cytoplasmic tail of TRAP in *P. falciparum* and *P. berghei* links to the actin-myosin motor through aldolase (Buscaglia et al., 2003), and a similar protein, containing a TSR domain (MTRAP), may provide the same function in merozoites for invasion of erythrocytes (Baum et al., 2005).

The motor complex that drives entry of the invasive parasites of Apicomplexa has been studied most extensively

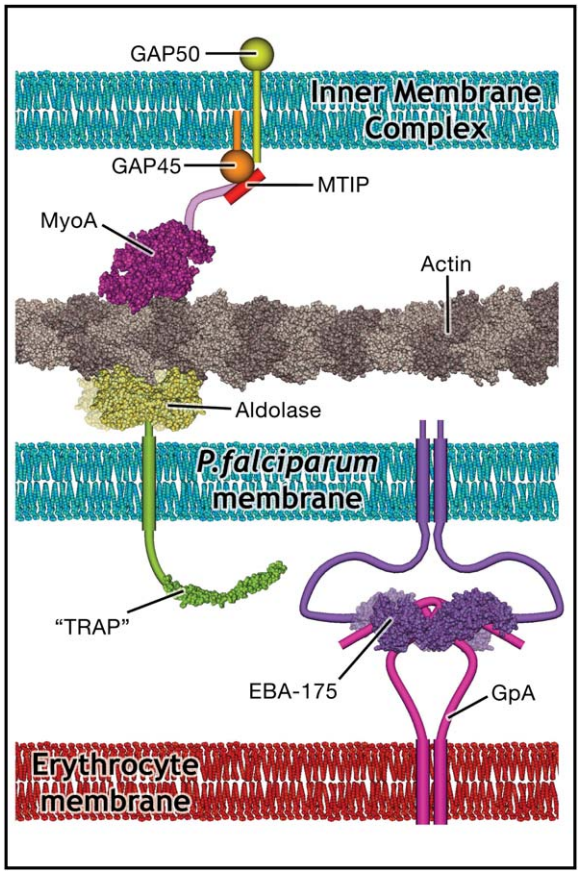


Figure 5. A Model for the Merozoite Motor and Associated Proteins

Key micronemal ligands such as EBA-175 bind to erythrocyte receptors, in this case glycophorin A (GpA). It is presumed that this complex is linked to the actin-myosin motor in some way; however, it seems unlikely that the C-terminal domain of EBA-175 is directly associated with the machinery. As in the *T. gondii* glideosome, it appears that a TRAP homolog, known as MTRAP, is present at the apical end of merozoites and may be linked to the motor via an interaction with aldolase. Whether MTRAP is indirectly involved in receptor binding via association with DBL or PfRh proteins remains to be determined. Whatever the case here, the bound surface proteins are linked to a motor complex that appears similar to that in *T. gondii* involving an aldolase interaction with F actin, which in turn is linked to the motor complex involving a myosin A (MyoA)-MTIP-GAP45 complex that associates with the transmembrane protein GAP50.

in *T. gondii*, and the proteins involved appear to be highly conserved across these organisms, including *Plasmodium* spp. (Baum et al., 2005). An unconventional class XIV of myosin A is linked to myosin light chain (MLC) (Opitz and Soldati, 2002), and this binds to the inner membrane complex (IMC) of the parasite pellicle via associated proteins called GAP50 and GAP45 (Gaskins et al., 2004). The homolog of MLC in *T. gondii* (Herm-Gotz et al., 2002) was identified in *P. yoelii*; it is known as MTIP and has been shown to bind to the equivalent MyoA protein (Bergman et al., 2003). This motor complex, which includes MyoA, MLC, GAP50, and GAP45, can be coprecipitated from the tachyzoite form of *T. gondii* (Gaskins et al., 2004), and a similar complex has also been identified in *P. falciparum* (Baum et al., 2005). Indeed, all apicomplexan parasites for which genome sequences are available encode homologs of GAP50, GAP45, MTIP/MLC, and MyoA, suggesting a highly conserved motor complex in these diverse parasites. This provides a general model of the motor machinery for Apicomplexa in which a receptor complex on the surface of the invasive parasite cell is linked to aldolase via a short cytoplasmic tail of a TRAP homolog (Figure 5). Aldolase binds filamentous actin (F actin), while the head region of MyoA can interface with these filaments. Force can be generated by the actin-myosin motor, anchored to the IMC and pellicle through MLC/MTIP, GAP50, and GAP45, allowing the invasive form of the parasite to be propelled forward and also allowing movement of the adhesin-aldolase-actin complex toward the posterior end of the invading cell. In order to release the adhesins from the host cell and allow forward movement, a protease appears to cleave the adhesins within the transmembrane regions in the parasite plasma membrane.

While the broad outline of this motor complex is understood, particularly in *T. gondii*, there are many questions that remain to be answered. Current evidence suggests that aldolase provides the link between the adhesins and F actin for *T. gondii* (Jewett and Sibley, 2003), and this needs to be firmly established for other parasites such as *P. falciparum*. It is also possible that proteins other than aldolase contribute to F actin binding that are yet to be determined. The movement generated by the motor complex of apicomplexan parasites is highly directional, and, consequently, the polymerization of G actin to F actin must be tightly regulated. Interestingly, F actin is difficult to detect in Apicomplexa parasites, probably because the filaments are short and perhaps because assembly and disassembly is rapid (Schmitz et al., 2005; Sahoo et al., 2006; Schuler et al., 2005). Indeed, analysis of G actin from *T. gondii* and *P. falciparum* has shown that it can polymerize but that the filaments formed are much shorter and less stable than that described for mammalian actin filaments. This suggests that there are differences in actin-filament dynamics for apicomplexan parasites, which may be explained by differences in protein sequence or in the properties of the actin regulatory proteins. Currently, there is little understanding of actin dynamics in the invasion process, and, with the availability of the genome sequence from many apicomplexan parasites, this will prove to be a fertile field in the future. Additionally, it may provide potential targets for the development of antiparasite drugs that interfere with the invasion process in a broad group of parasitic pathogens.

Future Perspectives

Malaria has plagued humans for many thousands of years, and, despite attempts to eradicate it through concerted programs of antimalarial drug treatment as well as vector control, the causative agent has adapted to these challenges. Major efforts are underway to develop new

antimalarial drugs and a vaccine. Because of its uniqueness, merozoite invasion of erythrocytes represents an attractive target for both endeavors. It is critical that we understand the role of different merozoite and host proteins in erythrocyte entry to provide a sound rational basis for the identification of vaccine and drug targets. Many important questions remain about erythrocyte invasion, just some of which have already been raised in this review: What mediates primary attachment? Are large complexes involved? What are the signals and mediators that link primary attachment to subsequent stages? Are conformational intermediates involved in receptor binding? How are the key secondary adhesins (EBAs and Rhs) linked to the motor? How is actin polymerization regulated?

Addressing functional issues such as these presents special problems, not the least of which relates to the requirement to perform molecular genetic studies in continuous blood-stage culture. Genetic manipulations that are deleterious to blood-stage development of *P. falciparum* are not possible at this stage. Hence, technological advances in *Plasmodium* are required, particularly as many important questions are *Plasmodium* specific and cannot be addressed in other systems. This will require improved systems for mutagenesis, both chemical and genetic, such as whole-genome transposon tagging (Balu et al., 2005). An important possibility now available with the use of conditional mutagenesis in *P. falciparum* (Meissner et al., 2005) is the ability to construct dominant-negative mutations and/or conditional deletions that effectively block the invasion process at specific points. Successful adaptation of this technology would not only allow the identification of the point at which the protein of interest plays its role, it would also lead to characterization of functionally important transient conformers and complexes. Such insights are likely to open many avenues for the development of novel intervention strategies.

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